REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
17-12-2015	FINAL	01-02-2012 to 30-09-2015

4. TITLE AND SUBTITLE
Investigations into the settlement and attachment of biofouling marine invertebrates

5a. CONTRACT NUMBER
N00014-12-1-0432

5b. GRANT NUMBER
n/a

5c. PROGRAM ELEMENT NUMBER
n/a

6. AUTHOR(S)
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5d. PROJECT NUMBER
15PR19595-00

5e. TASK NUMBER
n/a

5f. WORK UNIT NUMBER
n/a

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Cal Poly Corporation
1 Grand Avenue, Bldg. 15
San Luis Obispo, CA 93407

8. PERFORMING ORGANIZATION REPORT NUMBER
n/a

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Cal Poly Corporation
1 Grand Avenue, Bldg. 15
San Luis Obispo, CA 93407

10. SPONSOR/MONITOR'S ACRONYM(S)
n/a

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
n/a

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for Public Release; distribution is limited.

13. SUPPLEMENTARY NOTES
n/a

20151221053

14. ABSTRACT

Bugula neritina is a sessile marine bryozoan with a pelagic larval stage. Larvae frequently settle on boat hulls, facilitating the introduction of *B. neritina* to bays and estuaries worldwide. Adrenergic agonists, such as noradrenaline, inhibit larval settlement in a variety of marine invertebrate species, including *B. neritina*. Light also inhibits *B. neritina* larval settlement, yet the underlying mechanisms by which light and adrenergic compounds exert their effects on larvae are largely unknown. Octopamine is considered the invertebrate analog of noradrenaline, and may be involved in larval settlement pathways. In this study, we observed the effects of noradrenaline and the adrenergic antagonist phentolamine on larval settlement, and found that high concentrations of noradrenaline inhibited larval attachment and increased larval swimming behavior. High concentrations of phentolamine increased larval attachment and decreased larval swimming behavior. We used fluorescent labeling and microscopy to localize sensory system components, and found that larvae possess adrenergic-like receptors and octopamine-like immunoreactivity. We also exposed larvae to phentolamine in both dark and light conditions, and found that light inhibited larval attachment, but phentolamine blocked those inhibitory effects. Based on these results, we put forth a putative sensory pathway that explains the effects of both light and adrenergic compounds on *B. neritina* larval settlement behavior. This study sheds light on previously unknown larval sensory mechanisms and may aid in the development of effective, non-toxic biofouling control strategies.

15. SUBJECT TERMS
marine invertebrate larval settlement, *Bugula neritina*, octopamine, noradrenaline, phentolamine, adrenergic receptors, larval phototaxis, biofouling, investigation of larval sensory mechanisms

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	b. THIS PAGE			Wendt, Dean E.
U	U	U	UU	30	19b. TELEPHONE NUMBER *(Include area code)* 805-756-1508

CAL POLY
SAN LUIS OBISPO
CORPORATION

December 18, 2015

Defense Technical Information Center
8725 John J Kingman Road, Ste. 0944
Fort Belvoir, VA 22060-6218

Re. Award No. N00014-12-1-0432

Greetings,

Please find enclosed the Final Technical Report, with the SF298, for the above referenced grant.

If you have any questions or require further information, please do not hesitate to contact me by telephone at (805) 756-5348 or by email at lrebik@calpoly.edu.

Sincerely,

Leslie Rebik

Leslie Rebik
Contract & Grant Analyst

Enclosures

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Final Report (Award number: N000141210432)

Investigation of larval sensory systems in the marine bryozoan, *Bugula neritina*

Keywords and phrases: marine invertebrate larval settlement, *Bugula neritina*, octopamine, noradrenaline, phentolamine, adrenergic receptors, larval phototaxis, biofouling, investigation of larval sensory mechanisms

Abstract

Bugula neritina is a sessile marine bryozoan with a pelagic larval stage. Larvae frequently settle on boat hulls, facilitating the introduction of *B. neritina* to bays and estuaries worldwide. Adrenergic agonists, such as noradrenaline, inhibit larval settlement in a variety of marine invertebrate species, including *B. neritina.* Light also inhibits *B. neritina* larval settlement, yet the underlying mechanisms by which light and adrenergic compounds exert their effects on larvae are largely unknown. Octopamine is considered the invertebrate analog of noradrenaline, and may be involved in larval settlement pathways. In this study, we observed the effects of noradrenaline and the adrenergic antagonist phentolamine on larval settlement, and found that high concentrations of noradrenaline inhibited larval attachment and increased larval swimming behavior. High concentrations of phentolamine increased larval attachment and decreased larval swimming behavior. We used fluorescent labeling and microscopy to localize sensory system components, and found that larvae possess adrenergic-like receptors and octopamine-like immunoreactivity. We also exposed larvae to phentolamine in both dark and light conditions, and found that light inhibited larval attachment, but phentolamine blocked those inhibitory effects. Based on these results, we put forth a putative sensory

pathway that explains the effects of both light and adrenergic compounds on *B. neritina* larval settlement behavior. This study sheds light on previously unknown larval sensory mechanisms and may aid in the development of effective, non-toxic biofouling control strategies.

Introduction

Bugula neritina (Linnaeus 1758) is a sessile marine bryozoan with a pelagic larval stage, found in warm-temperate and subtropical waters worldwide (Ryland *et al.* 2011). *Bugula neritina* larvae frequently attach to boat hulls, and the species is regarded as one of the most widespread fouling bryozoans. A cosmopolitan distribution was reported for *B. neritina* as early as the 18th century, and shipping likely played a role in its introduction to bays and estuaries around the globe (Winston and Woollacott 2008). DNA sequencing of the mitochondrial gene cytochrome c oxidase I suggests that *B. neritina* is actually a complex of three cryptic species (Mackie *et al.* 2006, Davidson & Haygood 1999), which may have distinct native ranges (Fehlauer-Ale *et al* 2014). Native and non-native boundaries for *B. neritina* therefore remain unclear, but the range of the bryozoan is expanding (Winston and Woollacott 2008). (From this point on, *B. neritina* will be used to refer to the species complex, or *sensu lato* definition of the organism.) Increased knowledge of larval sensory mechanisms in fouling organisms like *B. neritina* will allow us to better understand factors that are responsible for their success as invasive species, and will enable us to develop improved strategies for preventing biofouling and further anthropogenic transport of non-native species to coastal ecosystems worldwide.

Many aspects of reproduction and development in *B. neritina* are well documented (e.g., Lynch 1947, Woollacott and Zimmer 1971). Adult colonies are comprised of branching, hermaphroditic zooids, and are typically brown to dark purple in color. Sexually reproduced embryos are brooded in modified zooids called ovicells, which release larvae that are non-feeding (aplankotrophic, Wendt 1996) and typically spend less than 24 h as plankton prior to settling (e.g., Wendt and Woollacott 1999). Larvae swim through the water column using cilia that cover most of the surface of their barrel-shaped bodies, collectively referred to as the ciliated corona (Woollacott and Zimmer 1971). Larvae often swim in a spiraling motion, and hold sensory structures in advance as they move through the water and begin exploration of a substratum. The sensory apical disc is located at the narrower end of the body, surrounded by a crown of rigid cilia and a circular cleft called the pallial furrow (Fig 1). The vibratile plume is another larval sensory structure, which consists of three long cilia that extend from the glandular pyriform groove (Fig. 1). Prior to attachment, larvae alight on a surface and spin counter-clockwise for 5-10 min, actively feeling the substratum with the vibratile plume (Lynch 1947). All visible cilial activity then halts for a brief moment prior to eversion of the internal sac, at which point metamorphosis is initiated and the animal is permanently attached to the substratum. The newly attached morph consists of the progenitor zooid, or ancestrula, which gives rise to all other zooids in the colony *via* asexual budding (Lynch 1947). Colonies can become reproductive and release larvae within just twelve days of metamorphosis (Wendt 1998).

While many aspects of *B. neritina* larval anatomy and behavior are well documented, the underlying sensory pathways that control larval settlement remain largely unknown. Literature on the effects of fouling-deterrent compounds on larval settlement in marine

75 invertebrates can provide insight into these pathways. Adrenergic compounds, such as the
76 hormone noradrenaline, inhibit larval settlement in a variety of marine invertebrates,
77 including *B. neritina* (e.g., Gohad *et al.* 2012, Shimizu *et al.* 2000). Noradrenaline (NA) is a
78 monoamine that binds to vertebrate adrenergic receptors and exerts a range of stimulatory
79 effects on the sympathetic nervous system, including increased heart rate, release of
80 glucose to the bloodstream, and increased blood flow to skeletal muscle. The underlying
81 mechanism by which NA exerts it effects on larval settlement in marine invertebrates is not
82 well understood, but one study on barnacle (*Balanus amphitrite*) cyprid larvae revealed the
83 presence of adrenergic-like receptors in sensory setae on antennules. These adrenergic-
84 like receptors may be the binding sites for NA and other adrenergic compounds (Gohad *et*
85 *al.* 2012). Octopamine is considered the invertebrate analog of NA and the two compounds
86 only differ structurally by the addition of one hydroxyl group to the benzene ring in NA
87 (Fig. 2).

88 Octopamine regulates a variety of physiological and behavioral processes ranging
89 from locomotion to photosensitivity in phylogenetically diverse invertebrates (Roeder
90 1999). Octopamine receptors have therefore been proposed as binding sites for adrenergic
91 compounds in invertebrates (Wendt *et al.* 2013). In *B. neritina,* octopamine receptors may
92 be the binding sites for NA and other adrenergic compounds, and endogenous octopamine
93 may be a neuroactive compound that modulates many aspects of larval behavior; including
94 locomotion, settlement, and phototaxis.

95 Photosensory systems play an important role in *B. neritina* larval behavior. Larval
96 release is induced in the laboratory by exposing dark-acclimated adult colonies to light (e.g.
97 Woollacott and Zimmer 1971, Wendt 1996), and continued light exposure inhibits larval

98 settlement (Wendt 1996). Larvae are photopositive upon release, but switch to become
99 photonegative within several hours (Lynch 1943, Wendt and Woollacott 1999). As they
100 move away from light, larvae begin the process of surface exploration that occurs prior to
101 attachment and metamorphosis. Thus, there is an inverse relationship between positive
102 phototaxis and initiation of metamorphosis (Wendt and Woollacott 1999). While the
103 effects of light on *B. neritina* larvae are well documented, the underlying sensory pathways
104 controlling these phenomena are still not well understood. One study investigated the
105 mechanisms underlying phototaxis by exposing *B. neritina* larvae to the monoamines
106 dopamine and serotonin. Dopamine exposure extended the period of positive phototaxis,
107 while serotonin, or 5-hydroxtryptophan (5HT), made larvae immediately photonegative.
108 5HT-like activity was also found in tracts connecting eyespots to the larval locomotory
109 organ (Pires and Woollacott 1997).

110 The metabolic pathways that involve dopamine are well studied in vertebrates.
111 Tyrosine is first converted by the enzyme tyrosine hydroxylase (TH) into L-DOPA, which is
112 then converted into dopamine. Dopamine is the precursor to several other monoamines,
113 including NA and octopamine (Fig 2). Therefore, it is possible that light exerts its effects on
114 *B. neritina* larvae *via* an underlying chemical pathway that involves both dopamine and
115 octopamine.

116 In the present study, *B. neritina* larvae were exposed to various concentrations of
117 the adrenergic agonist, NA, and the adrenergic antagonist, phentolamine, to investigate the
118 effects of these compounds on larval attachment, behavior, and mortality. Fluorescent
119 labeling and microscopy were used to determine the presence and location of adrenergic-
120 like receptors, octopamine, and tyrosine hydroxylase. Larvae were also exposed to light in

121 the presence and absence of phentolamine to observe their combined effects on larval
122 attachment and to gain knowledge of the underlying photosensory pathway.
123 We hypothesized that: 1. exposure to noradrenaline inhibits *B. neritina* larval
124 attachment, while exposure to phentolamine induces larvae to attach, 2. *B. neritina* larvae
125 possess adrenergic-like receptors, which serve as the binding sites for noradrenaline,
126 phentolamine, and other adrenergic-like compounds, 3. *B. neritina* larvae possess
127 endogenous octopamine, as well as the tyrosine-hydroxylase enzyme, which are located in
128 regions involved in the underlying pathway controlling larval settlement behavior, 4.
129 larvae exposed to phentolamine and light simultaneously will have elevated levels of
130 attachment as compared to those only exposed to light.

131 **Materials and methods**
132
133 *Larval collection*

134 *Bugula neritina* colonies were collected by hand from floating docks in two separate
135 locations in Morro Bay, CA USA (35.3708, -120.8580; 35.3461, -120.8432) from March 19,
136 2014 through May 1, 2015. Colonies were maintained in captivity in a dark, aerated
137 container of raw seawater at 11°C for 2 to 10 days, and given no exogenous food source. In
138 order to induce larval release, dark-acclimated colonies were exposed to light (both natural
139 and incandescent). Larvae were then collected and transferred by pipette within 1 h of
140 release. Larvae were pooled from multiple colonies to foster genetic heterogeneity for all
141 experiments.

142 *Effects of noradrenaline and phentolamine on larval attachment and behavior*

143 To observe the effects of NA (an adrenoreceptor agonist) and phentolamine (an
144 adrenoreceptor antagonist) on larval behavior and attachment, larvae were exposed to

145 varying concentrations of each compound in seawater and observed with a Leica EZ4D
146 dissecting microscope. DL-Noradrenaline Hydrochloride (≥97%) was obtained from Sigma-
147 Aldrich (St. Louis, MO, USA). Phentolamine-Hydrochloride (≥98%) was obtained from
148 Santa Cruz Biotechnology (Dallas, TX, USA). Larvae were released from multiple adult
149 colonies (collected on three different days from two Morro Bay sites), pooled in a beaker,
150 and transferred to 15 ml Falcon tubes with filtered seawater containing 0 (control), 0.1, 1.0,
151 10, or 100 μM of either NA or phentolamine.

152 Larvae were immediately transferred from Falcon tubes to 24 well polystyrene cell
153 culture plates, one larva per well in 1 ml of solution, with a total of twelve larvae per
154 treatment per trial. Five trials were conducted with both compounds (on the 15, 17, 22, 24,
155 and 29 of April, 2014), for a total of 600 larvae used in the experiment. Fresh treatment
156 solutions were made up for each trial. The number of larvae attached was recorded at 2, 4,
157 6, 8, 24, and 48 h following the start of treatment solution exposure. Larvae were
158 designated as attached if they had settled on the polystyrene and could not be moved by a
159 pipetted stream of water, or if they had settled on the air-water interface and begun to
160 metamorphose. Larvae were kept at 11°C for the duration of the experiments, except
161 during observation. Notes were also taken on larval behavior prior to attachment, and
162 unattached larvae were classified as swimming, spinning, or dead.

163 *Effects of noradrenaline and phentolamine on larval mortality*

164 To specifically determine whether experimental exposure to exogenous NA and
165 phentolamine solutions had an impact on larval mortality, larvae were exposed to
166 concentrations of 0 (control), 10, or 100 μM of NA or phentolamine in 65 mm diameter
167 petri dishes. These concentrations were based on results from unpublished pilot

experiments, and petri dishes were used to minimize larval mortality that may have occurred due to smaller wells of cell culture plates. Two trials were conducted, with ten larvae per treatment per trial, for a total of sixty larvae. The number of dead larvae was recorded at 24, 48, and 72 h of exposure. Mortality was assessed by lack of cilial movement in unattached larvae, and discoloration and/or termination of metamorphosis in attached larvae.

Localization of adrenergic-like receptors

Fluorescent labeling and microscopy were used to determine the presence and location of adrenergic-like receptors within whole mount *B. neritina* larvae. Live larvae were incubated in 10 μM BODIPY-FL Prazosin, a fluorescently labeled non-subtype selective α-adrenergic receptor antagonist obtained from Life Technologies (Foster City, CA, USA). The solution was made up in filtered seawater (FSW) and larvae were incubated for 30 min prior to being washed three times in FSW. Larvae were then imaged on an Olympus (Center Valley, PA, USA) BX53 compound fluorescent microscope with a DP73 camera, using the 488 nm laser line and Olympus CellSens software. Unstained larvae were used as controls for autofluorescence.

Localization of anti-octopamine and anti-tyrosine hydroxylase-like immunoreactivity

To determine the presence and location of tyrosine-hydroxylase-like and octopamine-like immunoreactivity, *B. neritina* larvae were fixed in 4% paraformaldehyde made up in phosphate-buffered saline (PBS) for at least 2 h, then washed once in PBS. Fixed larvae were then permeabilized overnight in 0.5% Triton X-100 in PBS, and incubated in 2% bovine serum albumin (BSA) for 4 h. BSA was pipetted off, and larvae were incubated in either anti-tyrosine hydroxylase primary antibody (1:500) or anti-octopamine primary

antibody (1:500) in PBS overnight at 4°C. Anti-tyrosine hydroxylase was obtained from Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA) and anti-octopamine was obtained from Millipore (Billerica, MA, USA). Larvae were washed in 0.1% Triton X-100 in PBS four times (5 min each) and those incubated with anti-tyrosine hydroxylase were transferred to Alexafluor 568 anti-mouse secondary antibody (1:500), while those incubated in anti-octopamine were transferred to Alexafluor 594 anti-rabbit secondary antibody (1:500) and incubated overnight at 4°C. Both secondary antibodies were obtained from Life Technologies. Larvae were incubated with the nuclear stain DAPI for 15 min (1:500), prior to washing four times (5 min each) in 0.1% Triton-X in PBS. Larvae were then imaged in PBS in chambered coverglass with an Olympus FV1000 Scanning Laser Confocal Microscope using Fluoview imaging software. Unstained larvae and larvae labeled with only secondary antibodies were used as controls for autofluoresence and unspecific binding, respectively.

Combined effects of light and phentolamine on larval attachment

To investigate underlying photosensory mechanisms in *B. neritina* larvae, larval attachment rates were compared between the following groups: 1. larvae immersed in 100 μM phentolamine and exposed to light, 2. larvae immersed in 100 μM phentolamine and kept in the dark, 3. larvae in FSW exposed to light, and 4. larvae in FSW kept in the dark. Larvae were collected from multiple colonies, pooled, and randomly transferred to 15 ml falcon tubes containing either FSW (control) or 100 μM phentolamine (treatment) in FSW. Each tube was transferred to a separate 65 mm diameter petri dish, and placed in either dark or light conditions for 4 hours at room temperature. The number of larvae attached

215 was recorded at 1, 2, and 3 h of exposure. Three trials were conducted, with ten larvae per
216 treatment per trial, for a total of 120 larvae.

217 *Statistical Analyses*

218 Binomial logistic regression and Tukey's HSD *post hoc* test were used to determine
219 whether rates of larval attachment and mortality differed significantly between treatment
220 and control groups. Nominal logistic regression and Cox Proportional Hazards risk ratios
221 were used to compare larval behavior between treatment and control groups. ANOVA and
222 Dunnett's test were used to determine whether rates of larval attachment differed
223 significantly between treatment and control groups in dark and light conditions. For
224 experiments that sampled individual larvae at multiple time points, separate analyses were
225 performed at each time point to avoid pseudo-replication. Residuals were normally
226 distributed, so data were not transformed prior to analyses. All statistical analyses were
227 conducted using JMP Pro 11 software (SAS, Cary, North Carolina, USA).

228 **Results**

229 *Effects of noradrenaline and phentolamine on larval attachment*

230 Binomial logistic regression revealed that noradrenaline (NA) significantly inhibited
231 *B. neritina* larval attachment at 10 μM (P=0.0297) and 100 μM (P=0.0121), but had no
232 significant effect at 0.1 or 1.0 μM (Fig. 3). In this experiment, the effects of phentolamine
233 on larval attachment were not statistically significant at any concentration. However, a
234 greater percentage of larvae in the 10 and 100 μM treatments attached, while a smaller
235 percentage of larvae in the 0.1 and 1.0 μM concentrations attached compared with the
236 control group (Fig 3). *Post hoc* pairwise comparisons (Tukey's HSD) revealed significant
237 differences between the control group and 100 μM NA at 2 h (P=0.0066), 4 h (P =0.0284), 6

238 h (P =0.0090), and 8 h (P =0.0111) of exposure; between the control group and 10 μM NA
239 at 2 h of exposure (P=0.0190); and between 100 μM NA and 100 μM phentolamine at 2 h (P
240 =0.0008), 4 h (P =0.0006), 6 h (P =0.0003), and 8 h (P =0.0010) of exposure (Fig. 3).
241
242
243 *Effects of noradrenaline and phentolamine on larval behavior*
244 We examined larval behavior at 2, 4, 6, and 8 h of exposure to either NA or
245 phentolamine, and classified each live, unattached larva as either swimming or spinning.
246 Nominal logistic regression and Cox Proportional Hazards risk ratios were used to compare
247 each treatment to the control at every time point. After 2 h of exposure, significantly more
248 larvae were swimming in 10 μM (P<0.0001) and 100 μM (P=0.0027) NA compared to the
249 control group, while significantly fewer larvae were swimming in 0.1 μM (P=0.0097) and
250 100 μM phentolamine (P<0.0001) (Fig. 4). After 4 h of exposure, significantly more larvae
251 were spinning in 10 μM (P<0.0001) and 100 μM (P<0.0001) NA, significantly more larvaew
252 were swimming in 100 μM NA (P =0.0058), and significantly fewer larvae were swimming
253 in 10 μM (P =0.0058) and 100 μM (P<0.0001) phentolamine. After 6 h of exposure,
254 significantly more larvae were swimming in 10 μM NA (P=0.0105), while significantly more
255 larvae were spinning in 100 μM NA (P=0.0002). Significantly fewer larvae were swimming
256 in 10 μM (P=0.0002) and 100 μM (P=0.0056) phentolamine. After 8 h, significantly more
257 larvae remained swimming in 10 μM NA (P=0.0234) and significantly more larvae
258 remained spinning in 100 μM NA (P=0.0013). Significantly fewer larvae remained
259 swimming in 10 μM (P<0.0001) and 100 μM (P<0.0001) phentolamine.

Effects of noradrenaline and phentolamine on larval mortality

Noradrenaline significantly increased larval mortality over a 72 h period at both 10 μM ($P<0.0001$) and 100 μM ($P<0.0001$). Phentolamine significantly increased larval mortality over a 72 h period at 100 μM ($P<0.0001$).

Localization of adrenergic-like receptors

Fluorescent signals detected in larvae stained with BODIPY-FL Prazosin indicate that *B. neritina* larvae do possess adrenergic-like receptors, which appear to be concentrated in and around the apical disc, as well as the pyriform groove (Fig. 5). No fluorescence was observed in control larvae that were not stained with BODIPY-FL Prazosin.

Localization of anti-octopamine and anti-tyrosine hydroxylase-like immunoreactivity

Both tyrosine hydroxylase-like and octopamine-like immunoreactivity were detected in *B. neritina* larvae, though some distortion of the larvae made determination of the precise locations of these substances difficult. Tyrosine hydroxylase-like immunoreactivity appeared to be concentrated in the apical disc, and the neuromuscular ring (Fig. 6). Octopamine-like immunoreactivity appeared to be most prevalent in the apical disc, the ciliated corona, and the pyriform groove (Fig. 7). No fluorescence was observed in control larvae that were not stained with primary antibodies.

Combined effects of light and phentolamine on larval attachment

Light exposure significantly inhibited larval attachment at the 1 h, 2 h, and 3 h exposure time points ($P<0.0001$). Phentolamine-exposed larvae in dark conditions also had significantly higher rates of attachment than control larvae in dark conditions after 1 h

284 (P=0.0004) and 2 h (P=0.004) of exposure. There was a significant interaction between
285 light and phentolamine after three hours of exposure, and the inhibitory effects of light
286 were significantly diminished in larvae exposed to 100 μM phentolamine (P=0.02) (Fig. 8).
287
288 **Discussion**
289 *Effects of noradrenaline and phentolamine on larval attachment, behavior, and mortality*
290
291 Our results confirm previous reports of the inhibitory effects of the adrenergic
292 agonist NA on the larval attachment of marine invertebrates (e.g. Shimizu *et al.* 2000,
293 Gohad *et al.* 2012). We expected an adrenergic antagonist to have the opposite effect, and
294 though not statistically significant, higher concentrations of phentolamine increased larval
295 attachment in our initial experiment (Fig. 3). Later experiments examining the effects of
296 both light and phentolamine allowed us to confirm this trend of increased attachment in
297 the highest concentration of phentolamine. Larvae exposed to 100 μM phentolamine had
298 significantly higher rates of attachment than control larvae in both light and dark
299 conditions (Fig. 8). These results on the effects of phentolamine on *B. neritina* larval
300 attachment contradict those from an experiment conducted by Dahms *et al.* (2004); a
301 discrepancy that may be explained by the method of counting attached larvae. More than
302 25% of attached larvae in the phentolamine treatments settled and began metamorphosis
303 on the air-water interface, never attaching to the polystyrene container. We counted these
304 larvae as attached, and considering them as unattached would have significantly altered
305 the results of the experiment.
306 That NA, an adrenergic receptor agonist, inhibited larval attachment at 100 μM,
307 while phentolamine, an adrenergic receptor antagonist, augmented larval attachment at

100 μM, supports our initial hypotheses and suggests that larvae do possess adrenergic-like receptors that are involved in the chemosensory pathways underlying settlement.

Analyses of *B. neritina* larval behavior in response to NA and phentolamine offer further insight into the mechanism by which these chemicals exert their effects. Our analysis of larval behavior was complicated by the fact that larvae can alternate between swimming and spinning during the period of surface exploration prior to attachment, and future analyses comparing cilial activity (in both swimming and spinning larvae) between treatment and control groups would provide further insight into the mechanisms underlying the effects of adrenergic compounds. However, our analysis revealed that NA (at 10 and 100 μM) increased swimming behavior, while phentolamine (at 100 μM) decreased swimming behavior after just 2 h of exposure (Fig. 4), and this pattern continued through the 8 h sampling period. These findings suggest that NA has a stimulatory effect on *B. neritina* larval cilia, thus prohibiting larvae from entering into a period of quiescence often observed prior to attachment. In vertebrate vascular smooth muscle, NA causes an increase in intracellular Ca^{2+} levels (Godfraind 1976), which can thereby increase airway ciliary activity (Lansley and Sanderson 1999). In invertebrates, such as the mollusc *H. trivolvis*, Ca^{2+} also causes an increase in ciliary beat frequency (Christopher *et al.* 1996). In barnacle cyprid larvae, exposure to octopamine, the invertebrate analog of NA, results in concentration-dependent increases in intracellular Ca^{2+} levels and significantly increases the speed of leg kicking (Lind *et al.* 2010). Octopamine exposure also inhibits *B. neritina* larval settlement in a similar fashion to NA (Shimizu *et al.* 2000). In *B. neritina* larvae, we therefore propose that endogenous octopamine regulates ciliary activity, and that the adrenergic-like receptors which bind NA and phentolamine are octopamine receptors.

While multiple studies have looked at the effects of adrenergic compounds on marine invertebrate larval settlement (e.g. Gohad *et al.* 2012, Shimizu *et al.* 2000), few have examined their effects on metamorphic success or larval survival over a longer duration of time. In our study, both NA (at 10 and 100 μM) and phentolamine (at 100 μM) significantly increased larval mortality over a 72 h period, and many larvae that successfully attached died shortly thereafter. These results suggest that adrenergic agonists and antagonists do more than simply extend or abbreviate the duration of larval swimming, and highlight the need for research into the long-term effects of these compounds on a range of organisms and ecosystems prior to their widespread use as biofouling controls.

Localization of adrenergic-like receptors

Results from fluorescent labeling and microscopy provide more evidence for the presence of octopamine receptors and endogenous octopamine in *B. neritina* larvae. Localization patterns were expected in sensory structures, such as the apical disc and the vibratile plume. Images of larvae stained with the fluorescently-labeled adrenergic receptor antagonist BODIPY-FL Prazosin, indicate that larvae possess adrenergic-like receptors in and around the apical disc, and in the pyriform groove, which houses the vibratile plume (Fig. 5). The apical disc and the vibratile plume are both held in advance of larvae as they move through the water, and are the first structures to come into contact with a substratum (Lynch 1947). These have long been considered the primary sensory structures in *B. neritina* larvae, and the presence of adrenergic-like receptors beneath the apical disc and in the glandular region underlying the vibratile plume provides evidence for their sensory role.

Localization of anti-octopamine and anti-tyrosine hydroxylase-like immunoreactivity

Images of larvae stained with anti-tyrosine hydroxylase provide further evidence for the sensory role of these structures. Anti-tyrosine hydroxylase-like immunoreactivity localized to the apical disc, to the ciliated corona, and to cells that appear to form a network between these regions and the neuromuscular ring (Fig. 6). Tyrosine hydroxylase is responsible for converting tyrosine to L-DOPA, the precursor to many hormones, including NA and octopamine (Fig. 2), and tyrosine-hydroxylase-like immunoreactivity is therefore considered indicative of neuroactive tissue. As early as 1890, Prouho asserted that the apical disc, the ciliated corona, and the pyriform organ in bryozoan larvae were all connected by nervous tissue, and our results support his claim.

Images of larvae stained with anti-octopamine further support this hypothesis, and suggest that *B. neritina* larvae do possess endogenous octopamine. Octopamine-like immunoreactivity localized to the apical disc, the ciliated corona, the pyriform groove and to underlying networks that appear to connect these structures (Fig. 7). Control larvae stained with only secondary fluorescent antibodies did not exhibit any red fluorescence, indicating that results seen in larvae stained with both primary and secondary antibodies were not due to autofluorescence or unspecific binding. While the specific location of octopamine was difficult to determine from our images, the pattern of immunoreactivity we observed was consistent with our expectations. These results provide the first evidence for endogenous octopamine in a bryozoan. The presence of octopamine within *B. neritina* may provide an explanation for how adrenergic compounds like NA exert their effects on marine invertebrate larvae, even though NA might not be synthesized by these species themselves.

Combined effects of light and phentolamine on larval attachment

Our results confirm that light significantly inhibits larval attachment. The inhibitory effect of light was significantly diminished when adrenergic-like receptors were blocked by phentolamine (Fig. 8), suggesting that these receptors are involved in the photosensory pathway. It is likely that these receptors are octopamine receptors, and that octopamine plays a role in controlling phototaxis in *B. neritina* larvae. In locusts (*Schistocerca gregaria*), the greatest density of octopamine receptors is found in optic lobes (Roeder and Nathanson 1993), and in honey bees (*Apis spp.*) exposure to octopamine increases positive phototaxis (Scheiner *et al.* 2014). Our results, combined with earlier work investigating the effects of dopamine and serotonin on *B. neritina* larvae (Pires and Woollacott 1997), suggest that dopamine and octopamine may both be involved in the photosensory pathway. Light may stimulate the production of dopamine, which is then converted to octopamine. While we did not investigate the role of serotonin in this study, it is possible that the hormone also plays a role in controlling *B. neritina* larval phototactic behavior.

Conceptual model of B. neritina larval sensory systems

Based on previous evidence and the results from the current study, we have constructed a conceptual model of some of the underlying sensory pathways that may control *B. neritina* larval behavior (Fig. 9). We propose that endogenously produced octopamine triggers an influx of calcium into cilial cells, stimulating their activity and resulting in swimming behavior. Exogenous exposure to adrenoreceptor agonists like NA results in stimulation of octopamine receptors, preventing larvae from becoming still and thereby inhibiting their attachment to a substratum. Conversely, exogenous exposure to adrenoreceptor antagonists like phentolamine can block octopamine receptors, preventing

larval swimming and causing larvae to attach more rapidly. Light exposure may naturally increase octopamine production within *B. neritina* larvae, stimulating the same pathway of increased cilial activity and settlement inhibition. This may also explain how light stimulates the release of larvae from the ovicells in which they are brooded, offering a mechanistic explanation for a phenomenon that has been observed and exploited in the laboratory for decades.

Future work

To further test this conceptual model (Fig. 9), video microscopy software could be used to calculate cilial beat frequencies and larval swimming speeds in *B. neritina* larvae exposed to light, octopamine, noradrenaline, and phentolamine. We predict that light and octopamine-exposed larvae would have significantly greater cilial beat frequencies and swimming speeds compared to control larvae, and that phentolamine-exposed larvae would have significantly lower cilial beat frequencies and swimming speeds. To gain more knowledge of the biosynthesis of endogenous octopamine, immunohistochemistry could be performed with an antibody targeting tyramine-beta hydroxylase, the enzyme that converts tyramine to octopamine (Fig 2). The presence of this enzyme is indicative of octopamine production, and would provide further information on the precise location of the hormone within *B. neritina* larvae. A comparison of octopamine levels between larvae exposed to light and kept in the dark using High Pressure Liquid Chromatography (HPLC) would shed further light on *B. neritina* larval photosensory pathways. We predict that larvae exposed to light would have significantly higher levels of endogenous octopamine compared with larvae kept in the dark. This experimental result would provide further evidence for our proposed hypothesis that light stimulates octopamine production.

Conclusion

Our investigations of *B. neritina* sensory systems offer insight into the underlying mechanisms controlling larval settlement behaviors that have been reported for decades, but not fully understood. An enhanced understanding of the larval biology of marine invertebrates like *B. neritina* not only expands our knowledge of the evolution of sensory system components across taxa, but can also aid in the development of new approaches to control biofouling and prevent the further spread of invasive species, which will benefit coastal ecosystems worldwide.

Acknowledgements

The monoclonal anti-tyrosine hydroxlase antibody was developed by Nicole Le Douarin and Catherine Ziller and was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, lowa City, lA 52242. All other antibodies were acquired from commercial distributors, as stated in the Materials and Methods.

This work would not have been possible without the guidance, expertise and support of our friends, colleagues, and mentors. ln particular, we would like to thank: Elena Keeling, Sean Lema, Lisa Needles, Ellen Calcagno, Alice Hamrick, Nancy Reid, Michael Curto, Lily Laiho, Lenora Brewer, Eric Vaught, Andrew Schaffner and the entire DEW lab (all from Cal Poly), and Randy Kleinen (from Olympus).

Literature Cited

Christopher, K., J. Chang, and J. Goldberg. 1996. Stimulation of cilia beat frequency by serotonin is mediated by a Ca^{2+} influx in ciliated cells of *Helisoma trivolvis* embryos. *J. Exp. Biol.* **199(5):** 1105-1113.

Dahms, H.U., T. Jin, and P.Y. Qian. 2004. Adrenoceptor compounds prevent the settlement of marine invertebrate larvae: *Balanus amphitrite* (Cirripedia), *Bugula neritina*

(Bryozoa) and *Hydroides elegans* (Polychaeta). *Biofouling* **20**: 313–321.

Davidson S.K. and M.G. Haygood. 1999. Identification of sibling species of the bryozoan *Bugula neritina* that produce different anticancer bryostatins and harbour distinct strains of the bacterial symbiont "Candidatus Endobugula sertula". *Biol. Bull.* **196**: 273–280.

Fehlauer-Ale, K. H., Mackie, J. A., Lim-Fong, G. E., Ale, E., Pie, M. R., and A. Waeschenbach. 2014. Cryptic species in the cosmopolitan *Bugula neritina* complex (Bryozoa, Cheilostomata). *Zool. Scripta* **43**: 193–205.

Godfraind, T. 1976. Calcium exchange in vascular smooth muscle, action of noradrenaline and lanthanum. *J. Physiol.* **260(1)**: 21-35.

Gohad, N., N. Aldred, B. Orihuela, A. Clare, D. Rittschof, and A. Mount. 2012. Observations on the settlement and cementation of barnacle (*Balanus amphitrite*) cyprid larvae after artificial exposure to noradrenaline and the locations of adrenergic-like receptors. *J. Exp. Mar. Biol. Ecol.* **416-417**: 153-161.

Lansley, A. and M. Sanderson. 1999. Regulation of Airway Ciliary Activity by Ca^{2+}: Simultaneous Measurement of Beat Frequency and Intracellular Ca^{2+}. *Biophys. J.* **77**: 629–638.

Lind U, R. Alm, F. Hasselberg, S. Falkbring, L. Brive, et al. 2010. Octopamine receptors from the barnacle *Balanus improvisus* are activated by the alpha2-adrenoceptor agonist medetomidine. *Mol. Pharmacol.* **78**: 237–248.

Lynch, W. R. 1947. The behavior and metamorphosis of the larva of *Bugula neritina* (Linnaeus): experimental modification of the length of the free-swimming period and the responses of the larvae to light and gravity. *Biol. Bull.* **92**: 115- 150.

Mackie, J., M. Keough, and L. Christidis. 2006. Invasion patterns inferred from cytochrome oxidase I sequences in three bryozoans, *Bugula neritina, Watersipora subtorquata,* and *Watersipora arcuata. Mar. Biol.* **149**: 285–295.

Pires, A. and R.M. Woollacott. 1997. Serotonin and dopamine have opposite effects on phototaxis in larvae of the bryozoan *Bugula neritina. Biol. Bull.* **192**: 399-409.

Prouho, H. 1890. Recherches sur la larve de *Flustrella hispida* (Gray) structure et metamorphose. Archives de Zoologie Expérimentale et Générale, 2nd Series, **8**:409-459.

Reed, C.G. 1987. Phylum Bryozoa viewed from the egg. Pp. 494-510 in *Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast,* M.F. Strathman, ed. University of Washington Press, Seattle and London.

Roeder, T. and J. A. Nathanson. 1993. Characterization of Insect Neuronal Octopamine Receptors (OA3 Receptors). *Neurochem. Res.* **18(8)**: 921-925.

Roeder, T. 1999. Octopamine in Invertebrates. *Prog. Neurobiol.* **59**: 553-561.

Ryland, J., J. Bishop, H. Blauwe, A. Nagar, D. Minchin, C. Wood, and A. Yunnie. 2011. Alien species of *Bugula* (Bryozoa) along the Atlantic coasts of Europe. *Aquat. Invasions* **6(1)**: 17–31.

Scheiner, R., Toteva, A., Reim, T., Sevik, E., and A. Barron. 2014. Differences in the phototaxis of pollen and nectar foraging honey bees are related to their octopamine brain titers. *Front. Physiol.* **116(5)**: 1-8.

Shimizu, K., E. Hunter, and N. Fusetani. 2000. Localisation of biogenic amines in larvae of *Bugula neritina* (Bryozoa: Cheilostomatida) and their effects on settlement. *Mar. Biol.* **136**:1-9.

Wendt, I., Å. Arrhenius, T. Backhaus, A. Hilvarsson, K. Holm, K. Langford, T. Tunovic, and H. Blanck. 2013. Effects of Five Antifouling Biocides on Settlement and Growth of Zoospores from the Marine Macroalga *Ulva lactuca L. Bull. Environ. Contam. Toxicol.* **91**: 426–432.

Wendt, D. E., and R. M. Woollacott. 1999. Ontogenies of phototactic behavior and metamorphic competence in larvae of three species of *Bugula* (Bryozoa). *Invertebr. Biol.* **118**: 75–84.

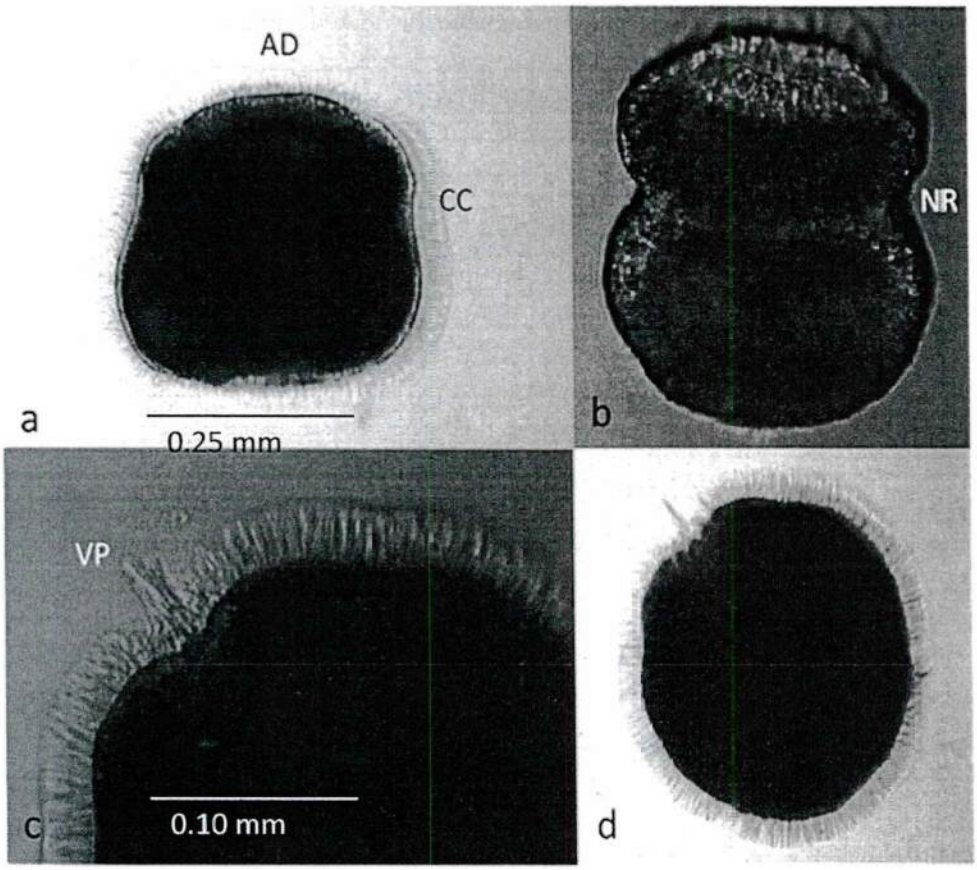
Wendt, D.E. 1998. Effect of larval swimming duration on growth and reproduction of *Bugula neritina* (Bryozoa) under field conditions. *Biol. Bull.* **195**: 126-135.

Wendt, D.E. 1996. Effect of larval swimming duration on success of metamorphosis and size of the ancestrular lophophore in *Bugula neritina* (Bryozoa). *Biol. Bull.* **191**: 224–233.

Winston, J.E., and R. M. Woollacott. 2008. Redescription and revision of some red-pigmented *Bugula* species. *Bull. Mus. Comp. Zool.* **159 (3)**: 179-212.

Woollacott, R. M., and R. L. Zimmer. 1975. A simplified placenta- like system for the transport of extraembryonic nutrients during embryogenesis of Bugula neritina. *J. Morphol.* **147**: 355-371.

Woollacott, R. M., and R. L. Zimmer. 1971. Attachment and metamorphosis of the cheilo-ctenostome bryozoan *Bugula neritina. J. Morphol.* **134(3)**: 351-382.



536
537

538 **Figure 1.** Brightfield images of *B. neritina* larvae taken with an Olympus BX53 microscope
539 and DP73 camera using CellSens software. **a.** Barrel-shaped larvae are surrounded by a
540 ciliated corona (CC), which acts as larval locomotory organ. The image was taken from the
541 lateral view, with the apical disc (AD) upmost. **b.** Larvae fixed with 4% paraformaldehyde
542 can become distorted and drop cilia. The neuromuscular ring (NR) underlies the
543 constricted region. **c.** This image taken from the apical view offers a closer view of the
544 ciliated corona and the vibratile plume (VP). **d.** The vibratile plume extends from the
545 pyriform groove, and the ciliated corona is distributed across the surface of the larva.

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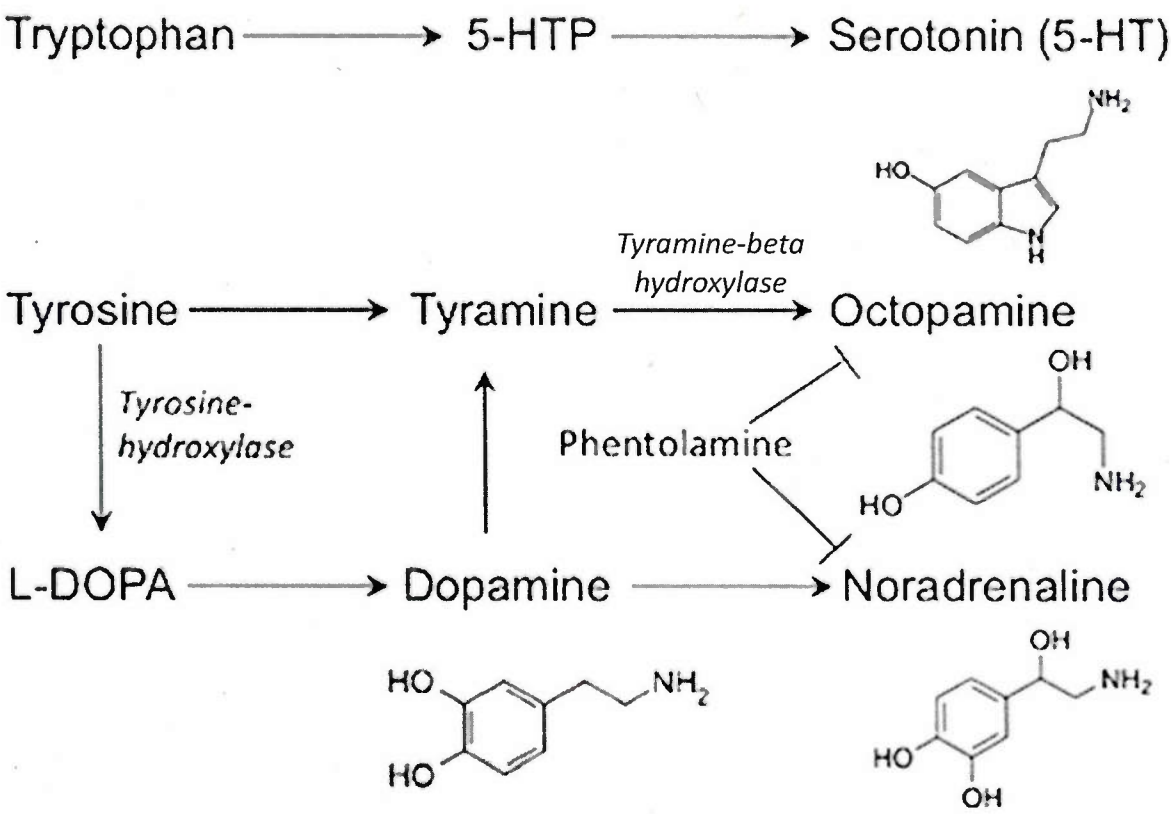
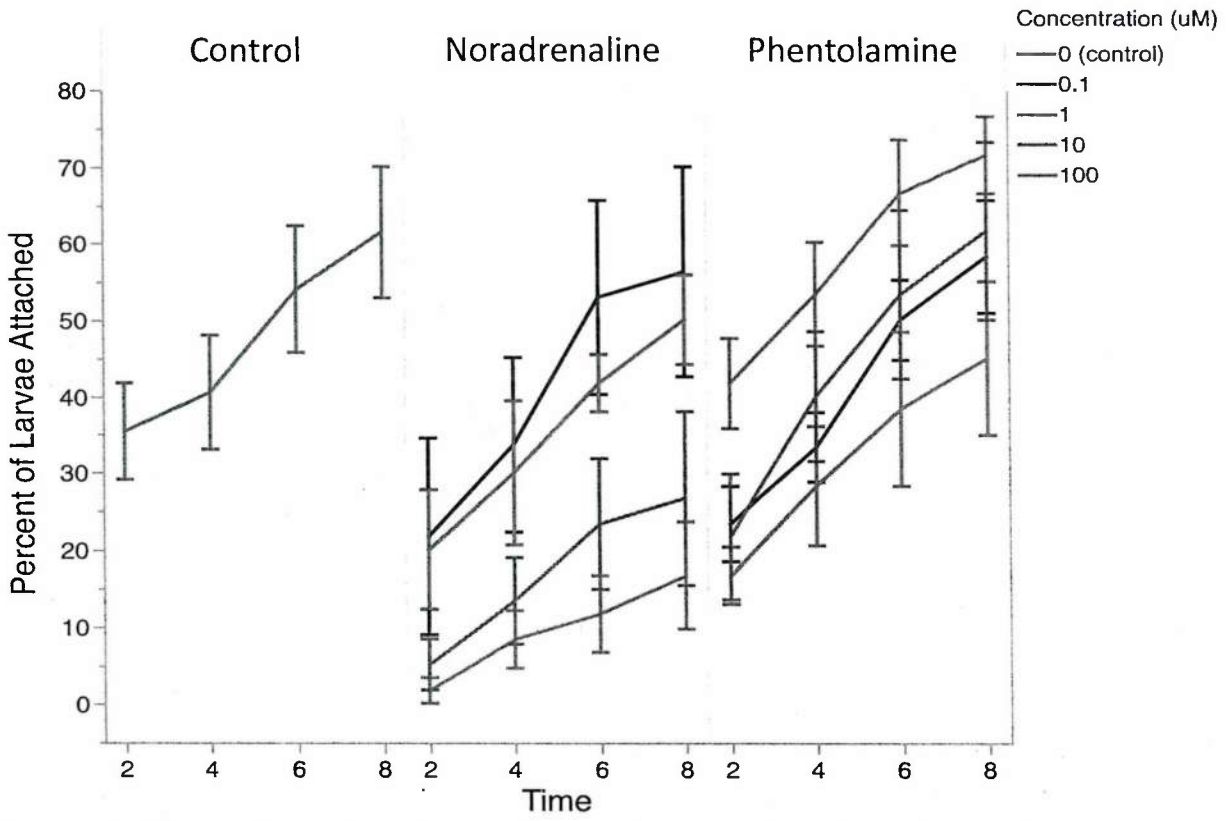
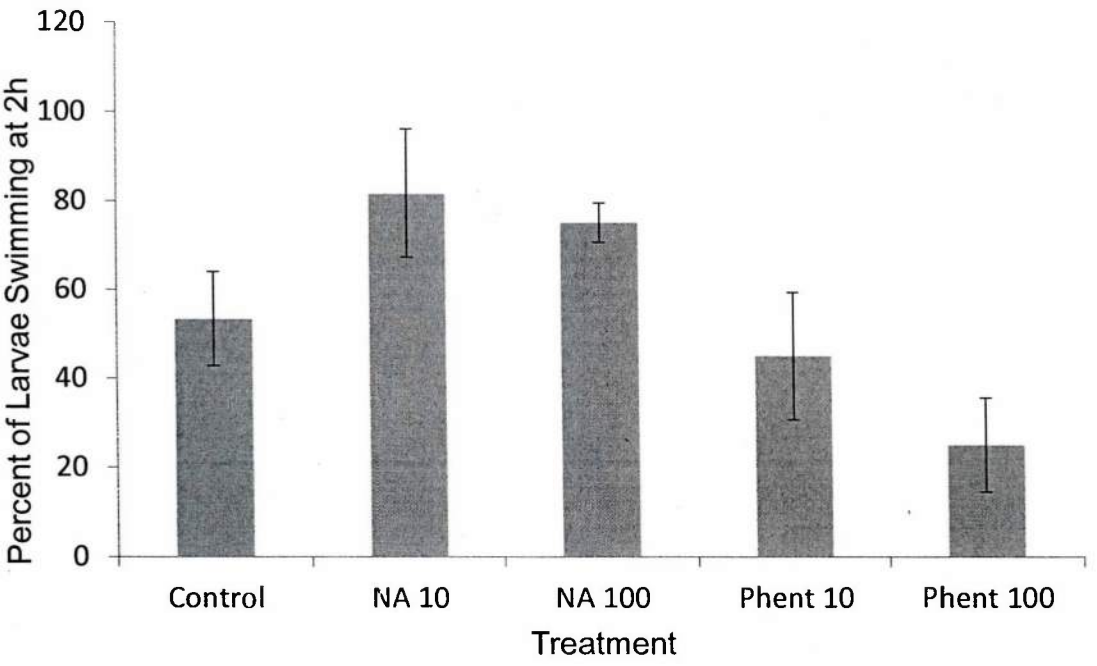


547
548 **Figure 2.** Biosynthesis of some of the neuroactive compounds likely involved in controlling
549 *B. neritina* larval behavior. Noradrenaline, an adrenergic receptor agonist, inhibits larval
550 settlement in many marine invertebrates, including *B. neritina.* Phentolamine is a
551 pharmaceutical compound that blocks adrenergic receptors. Octopamine is often
552 considered the invertebrate equivalent of noradrenaline, and both monoamines an be
553 synthesized from tyrosine and dopamine. Tyrosine hydroxlase is the enzyme that converts
554 tyrosine to L-DOPA (the precursor to dopamine). Dopamine and serotonin influence *B.*
555 *neritina* larval phototaxis.
556



557
558 **Figure 3.** Effects of noradrenaline and phentolamine on larval attachment over 8 h of
559 treatment exposure. Sample size was n=5 replicate trials, with 12 larvae per treatment per
560 trial, for a total of 60 larvae per treatment. Error bars represent standard errors of the
561 means. NA significantly inhibited larval attachment at 10 μM (P=0.0297) and 100 μM
562 (P=0.0121). The effects of phentolamine were not significant, but there was a trend of
563 lower attachment at 0.1 and 1.0 μM, and higher attachment at 10 and 100 μM.
564



565 **Figure**

566 **4.** Effects of noradrenaline and phentolamine on larval swimming behavior at 2 h of

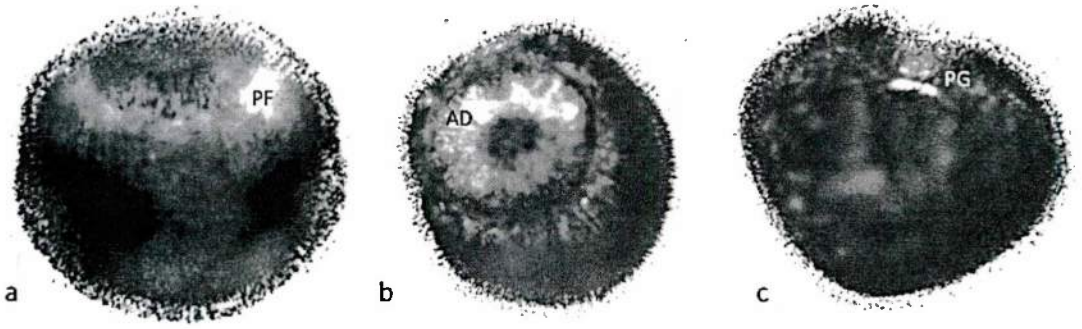
567 exposure. Sample size was *n*=5 replicate trials, with 12 larvae per treatment per trial, for a

568 total of 60 larvae per treatment. Error bars represent standard errors of the means. NA

569 significantly increased swimming behavior at 10 and 100 μM, while phentolamine

570 significantly decreased swimming behavior at 100 μM.

571



572 **Figure**

573 **5.** *B. neritina* larva stained with BODIPY-FL Prazosin, a fluorescently labeled adrenergic

574 receptor antagonist. Fluorescence was observed in: **a.** the region surrounding the apical

575 disc, **b.** the apical disc, and **c.** the pyriform groove, suggesting that larvae possess

576 adrenergic-like receptors in each of these regions. Images were taken with the 20X

577 objective on an Olympus BX53 microscope, using a DP73 camera and CellSens software.

578 The 488 nm laser line was used, and images were then converted to gray scale.

579

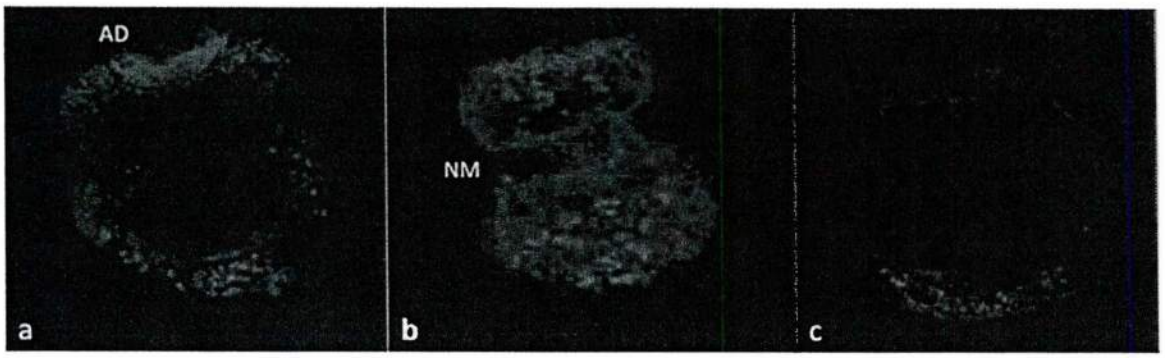


Figure 6. a) *B. neritina* larva stained with DAPI nuclear stain (blue), and anti-tyrosine hydroxylase (red). At the surface, tyrosine hydroxylase-like immunoreactivity localized to the apical disc region (AD). **b)** An image taken deeper inside the larva shows tyrosine hydroxylase-like immunoreactivity (red) in the region of the neuromuscular ring (NM). **c)** Control larva stained with only DAPI nuclear stain and secondary antibody. All images were taken on an Olympus FV1000 Scanning Laser Confocal Microscope using Fluoview imaging software and the 20X objective. A 405 nm laser line at 8% transmissivity was used for DAPI excitation (blue), and a 559 nm laser line at 12% transmissivity was used for Alexafluor-568 excitation (red). A 3D z-stack video file of this larva is provided in the supplemental materials.

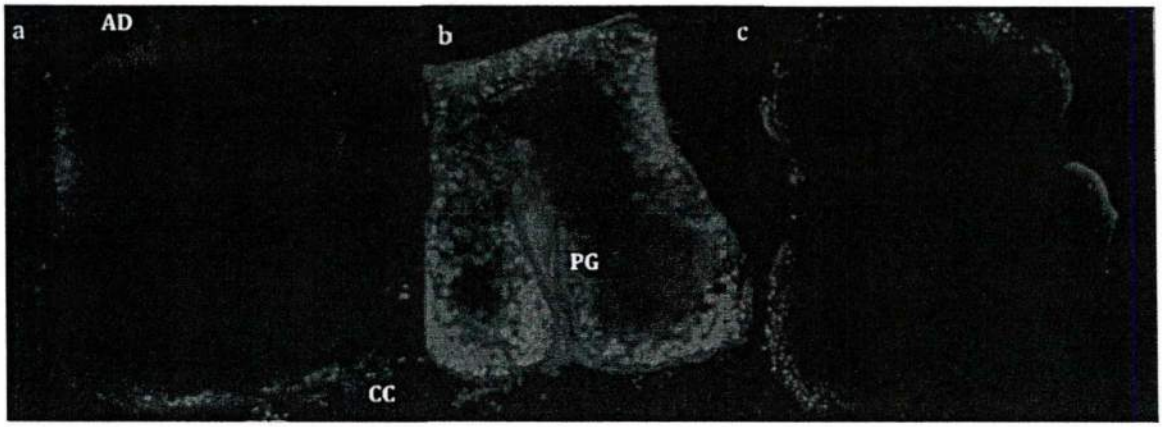
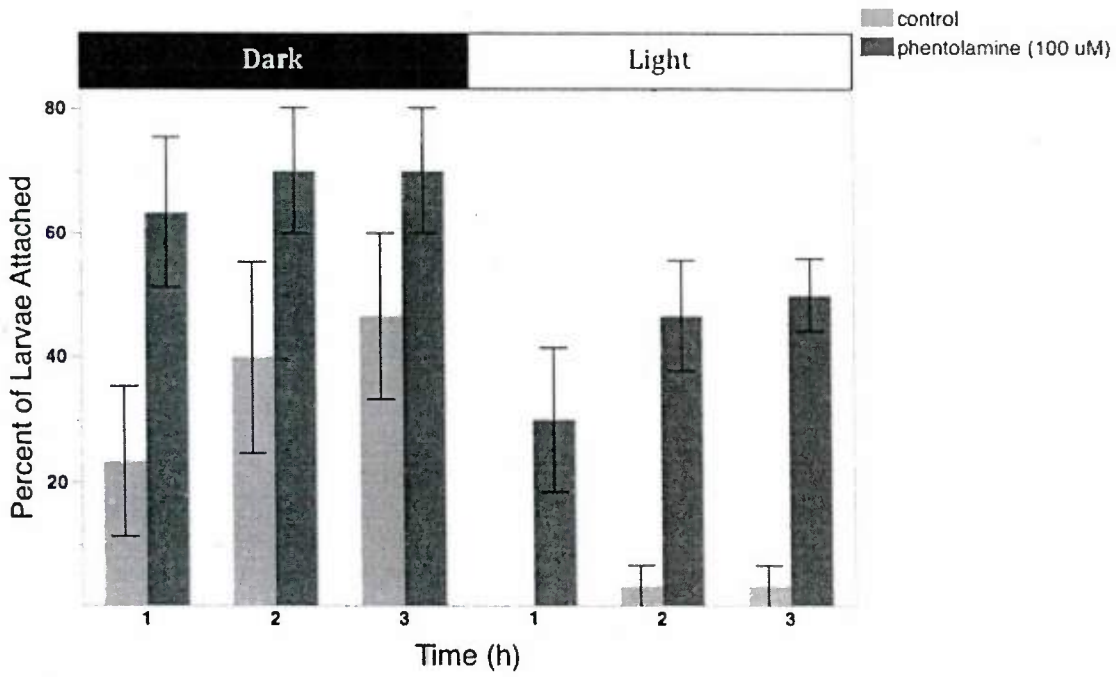


Figure 7. a) *B. neritina* larva stained with DAPI nuclear stain (blue), and anti-octopamine (red). At the surface, octopamine-like immunoreactivity localized to the apical disc (AD) and the ciliated corona (CC). **b)** An image from deeper inside the larva reveals octopamine-like activity in the region of the pyriform groove (PG), which houses the vibratile plume. **c)** Control larvae stained with only DAPI and secondary antibody. All images were taken on an Olympus FV1000 Scanning Laser Confocal Microscope using Fluoview imaging software and the 20X objective. A 405 nm laser line at 8% transmissivity was used for DAPI excitation (blue), and a 559 nm laser line at 30% transmissivity was used for Alexafluor-594 excitation (red). A 3D z-stack video file of this larva is provided in the supplemental materials.



606

607 **Figure 8.** Effects of light and phentolamine on larval attachment. Sample size of n=3

608 replicate trials, with 10 larvae per treatment per trial, for a total of 30 larvae per treatment.

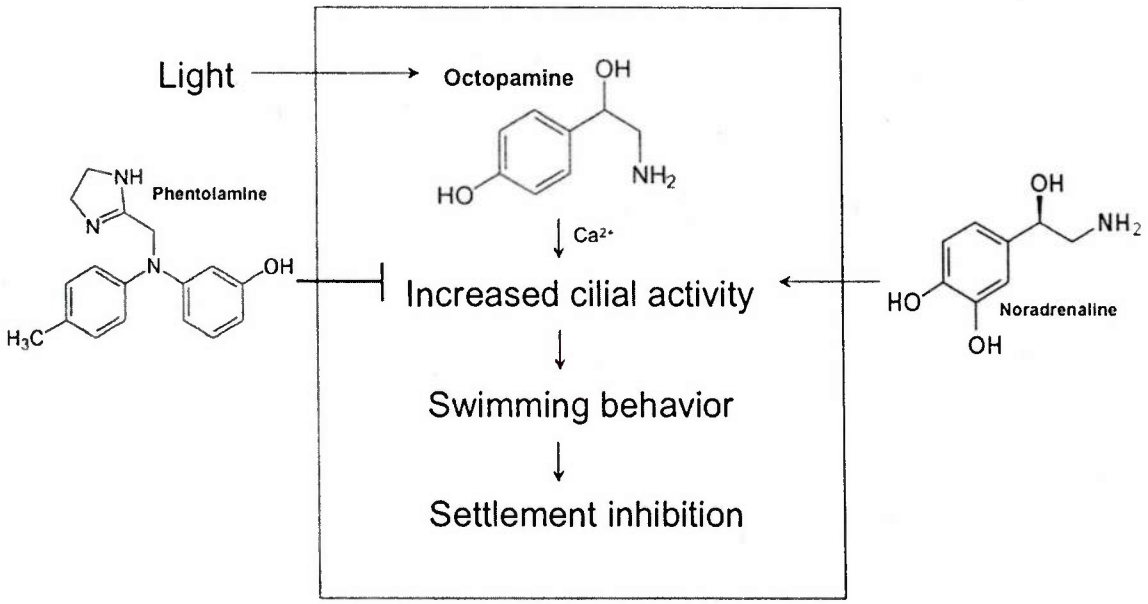
609 Error bars represent standard errors of the mean. Light significantly inhibited larval

610 attachment in control larvae at each timepoint . The effect of light was significantly

611 diminished by phentolamine (an adrenergic receptor blocker) after 3 h of exposure,

612 suggesting that adrer.ergic-like receptors play a role in the photosensory pathway.

613



614 **Figure**

615 **9.** Putative *B. neritina* larval sensory mechanism. Light stimulates octopamine production,

616 which thereby increases intracellular levels of Ca^{2+} and causes larval cilia to beat. Beating

617 cilia result in swimming or spinning behavior, which prevents larvae from entering into a

618 necessary period of quiescence prior to attachment. Noradrenaline binds octopamine

619 receptors, stimulating cilial activity, while phentolamine blocks octopamine receptors,

620 preventing larval swimming.

621